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A highly sensitive and selective immunoassay for the detection of tetrabromobisphenol A in soil and sediment

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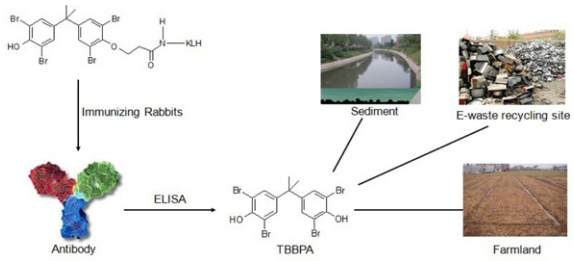
HIGHLIGHTS

- The developed ELISA is highly sensitive and selective to TBBPA.
- Accuracy of this ELISA for TBBPA in environmental matrices were reasonable.
- TBBPA levels found in environmental samples showed variation.
- ELISA for TBBPA in real samples correlated well with LC–MS/MS method.

GRAPHICAL ABSTRACT



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ABSTRACT

Tetrabromobisphenol A is the most widely used brominated flame retardant. A sensitive and selective enzyme-linked immunosorbent assay (ELISA) for the detection of tetrabromobisphenol A was developed. The limit of detection and the inhibition half-maximum concentration of tetrabromobisphenol A in phosphate buffered saline with 10% methanol were 0.05 and 0.87 ng mL^{-1}, respectively. Cross-reactivity values of the ELISA with a set of important brominated flame retardants including tetrabromobisphenol A-bis(2,3-dibromopropylether), 2,2′,6,6′-tetrabromobisphenol A diallyl ether, hexabromocyclododecane, 1,2-bis(pentabromodiphenyl) ethane, 1,2-bis(2,4,6 tribromophenoxy) ethane, bis(2-ethylhexyl)-3,4,5,6-tetrabromophthalate, 2-ethylhexyl-2,3,4,5-tetrabromobenzoate, and polybrominated diphenyl ethers were <0.05%. Concentrations of tetrabromobisphenol A determined by ELISA in the soils from farmlands, the soils from an e-waste recycling site, and the sediments of a canal were in the range of non-detectable–5.6 ng g^{-1}, 26–104 ng g^{-1} and 0.3–22 ng g^{-1} dw, respectively, indicating the ubiquitous pollution of tetrabromobisphenol A. The results of this assay for 16 real world samples agreed well with those of the liquid chromatography–tandem mass spectrometry method, indicating this ELISA is suitable for screening of tetrabromobisphenol A in environmental matrices.

1. Introduction

Tetrabromobisphenol A (TBBPA) has been the most widely used brominated flame retardant (BFR). TBBPA is mainly used as a reactive flame retardant, i.e. covalently bonded to the host materials in, for example, epoxys and polycarbonate resins in printed circuit boards and electronic equipment. It can also be mixed with the host materials as an additive flame retardant, for instance in high impact polystyrene and acrylonitrile-butadiene-styrene resins. The global consumption estimates of TBBPA were close to 120,000 tons in 2001, as the Asian countries registered the highest consumption of TBBPA (89,400 tons/year) followed by USA (18,000 tons/year) and the European countries (11,600 tons/year) [1]. The size of the global TBBPA market reported by the European BFR Industry Panel

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was 170,000 tons in 2004 [2]. The production capacity of TBBPA in China was reported about 18,000 tons in 2007 [3].

The European Union risk assessment of TBBPA on human health (Part II, 2006) concluded that there were no human health hazards of concern and no risks were identified [4]. However, there are indications about the potential toxicity of TBBPA as an endocrine disrupting [5], immunotoxic [6], and neurotoxic compound [7]. Other studies showed that it is toxic to aquatic life [8]. TBBPA could be dehalogenated under anaerobic [9] and aerobic [10] conditions to yield bisphenol A (BPA), a widely used compound that has been reported to be an endocrine disruptor [11].

TBBPA is transferred from different processes and sources to the environment. Trace concentrations have been detected both in abiotic and biotic samples [12,13]. Procedures used for the analyses of TBBPA and its derivatives in a wide variety of environmental samples have recently been reviewed by Covaci et al. [12]. Analyses are usually accomplished using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) techniques, by which TBBPA is measured with hexabromocyclododecane (HBCD) or other phenolic (halogenated) organic compounds in one run. Although the GC–MS method showed good separation properties, acidification and derivatization of TBBPA are required [14,15]. The LC–MS methods provide advantages by avoiding the derivatization. The use of ^{13}C-labelled TBBPA as an internal standard enhances the quality of the analytical data through compensation for matrix-related effects that can affect analyte ion intensity, trueness and reproducibility. The LC–tandem mass spectrometry (LC–MS/MS) appears to be the method of choice because of its high sensitivity and specificity [16,17]. Among the predominant BFRs, TBBPA is the most polar molecule, and thus alternate analytical methods may be applicable.

Enzyme-linked immunosorbant assays (ELISAs) having an established record of being sensitive, specific, and capable of high throughput would be useful in the monitoring of this environmental pollutant. To the best of our knowledge, there are no reported ELISAs available for the analysis of TBBPA. The positive features of ELISAs in measuring other BFRs such as polybrominated diphenyl ethers (PBDEs) have been well recognized [18–22]. We believed this approach could be useful for TBBPA. Thus, this study aimed to develop an ELISA for TBBPA as a quantitative screening procedure applied to environmental samples. The synthesis of diverse haptens useful to elicit antibodies against TBBPA is described. In addition, the new ELISA method is compared with a well established LC–MS/MS method for the analysis of TBBPA in environmental samples.

2. Experimental

2.1. Reagents and materials

All reagents were of analytical grade unless specified otherwise. *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-rabbit IgG horseradish peroxidase conjugate (IgG-HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), and 4,4-bis(3,5-dibromo-4-hydroxyphenyl)pentanoic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). TBBPA (99% purity) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Ring-$^{13}C_{12}$ labeled TBBPA (99% purity) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). BDE congeners and metabolites were purchased from AccuStandard (New Haven, CT).

Organic materials for the hapten synthesis were purchased from Sigma–Aldrich Chemical Co. and J&K Scientific Ltd. (Beijing, China). Column chromatographic separations were carried out using silica gel (40 μm average particle size) from Shanghai Sanpont Co., Ltd. (Shanghai, China) and the indicated solvents. Purities were confirmed with Sanpont thin layer chromatography (TLC) silica gel plates.

2.2. Instrumentation

ELISA was carried out in 96-well polystyrene microplates (Nalge Nunc International, Denmark) and absorbance values of microplate wells were determined with a microplate reader (Wellscan MK3, Labsystems Dragon, Finland). Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance DPX 300 MHz spectrometer system (BrukerBioSpin GmbH, Rheinstetten, Germany). Electrospray mass spectra of haptens in negative mode were recorded by an Agilent inert 6890/5973 mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA).

For the LC–MS/MS analysis of TBBPA, an Agilent 1200 series HPLC system equipped with an Agilent Eclipse Plus C_{18} column (2.1 mm × 50 mm, 3.5 μm) was used. The mobile phase was acetonitrile:water (9:1, v/v) at a flow rate of 0.3 mL min^{-1}. The injection volume was set at 10 μL. Mass spectrometric analysis was performed with an Agilent 6410B Triple Quad LC/MS system equipped with an electrospray ionization (ESI) Turbo spray interface. All data were acquired and processed using Agilent MassHunter Qualitative Analysis Software (version A.00.05.25). For target quantitative analyses, data acquisition was performed in the multiple reaction monitoring (MRM) mode. Selected ion monitoring of the deprotonated analyte ions $[M-H]^-$ was then performed in retention time scheduled events. The selected ions were m/z 542.8 > 445.8 for TBBPA and m/z 554.8 > 457.8 for $^{13}C_{12}$-TBBPA. The MS/MS detection conditions were optimized as Capillary, 3500 V; gas temperature, 300 °C; gas flow, 8 L min^{-1}; Nebulizer, 30 psi; Fragmentor, 220 V; Collision energy, 34 eV.

2.3. Hapten synthesis

Six haptens named Tn (n = 1–6) were employed in this study (Fig. 1). Five haptens were synthesized according to the routes illustrated in Fig. 1, whereas hapten T3 is commercially available. Synthesis procedures and characterization data of these compounds are given below.

3-(2,6-Dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl))propanoic acid (hapten T1): A solution of 3-chloropropionic acid (1.0 g, 9.2 mmol) and NaOH (0.37 g, 9.2 mmol) in 15 mL of water was added dropwise to a mixture of TBBPA (10 g, 18.4 mmol) and NaOH (1.5 g, 36.8 mmol) in 30 mL of water under stirring. After refluxing for 5 h, the mixture was cooled to ambient temperature. The solution was acidified to pH 1.0–2.0 with 37% HCl and then extracted with ethyl acetate (3 mL × 20 mL). The organic phase was washed with water (2 mL × 20 mL) and evaporated under vacuum. The residue was purified by flash silica column chromatography (ethyl acetate/petroleun ether, 1:4, v/v) to obtain 3-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl))propanoic acid (T1). Yield: 63%. ^{1}H NMR (DMSO) δ (ppm): 12.39 (1H, s, COO*H*), 9.86 (1H, s, O*H*), 7.45 (2H, s, Aromatic H), 7.38 (2H, s, Aromatic H), 4.18 (2H, t, J = 6.39 Hz, O$CH_2$$CH_2$COOH), 2.76 (2H, t, J = 6.40 Hz, OCH$_2$CH_2COOH), 1.59 (6H, s, –2CH_3). ^{13}C NMR (DMSO) δ (ppm): 171.86, 150.73, 149.11, 148.92, 143.45, 131.02, 130.48, 117.47, 112.02, 69.50, 41.81, 35.18, 29.93. MS-ESI: $[M-H]^-$ m/z = 614.8.

2-(2,6-Dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl))acetic acid (hapten T2): Ethyl bromoacetate (0.84 g, 5.0 mmol) dissolved in 15 mL of tetrahydrofuran was added dropwise to a mixture of TBBPA (3.27 g, 6.0 mmol) and NaOH (0.8 g, 20 mmol) in 20 mL of water under stirring. After refluxing for 5 h, the mixture was cooled to ambient temperature. The solution was

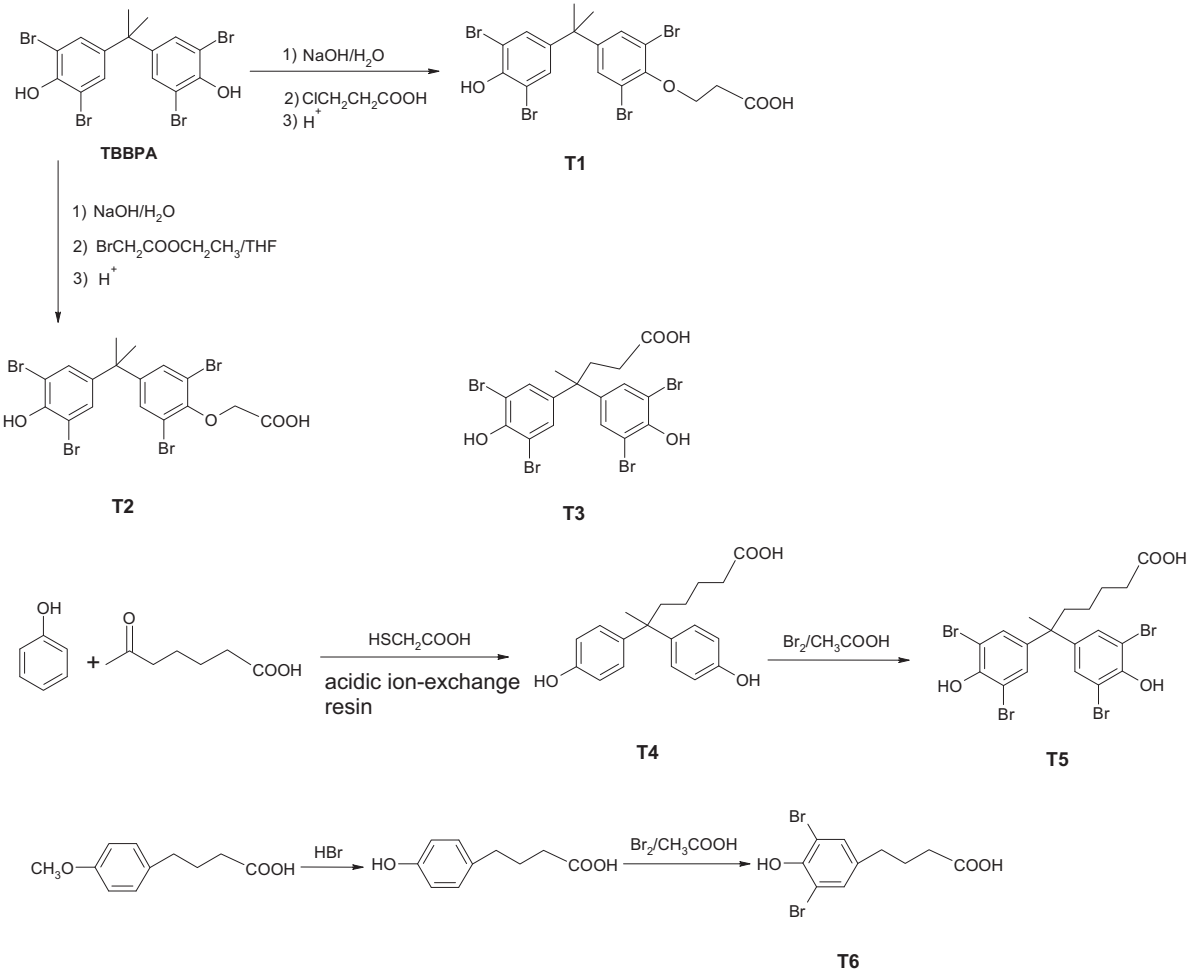

Fig. 1. Synthetic routes of different haptens (T1–T6) of TBBPA.

acidified to about pH 1.0 with 37% HCl and then extracted with ethyl acetate (3 mL × 20 mL). The organic phase was washed with water (2 mL × 20 mL) and evaporated under vacuum. The residue was purified by flash silica column chromatography (ethyl acetate/petroleun ether, 1:4, v/v) to obtain 2-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl))acetic acid (T2). Yield: 67%. ^{1}H NMR (DMSO) δ (ppm): 1.15 (1H, br, COO*H*), 9.85 (1H, s, O*H*), 7.47 (2H, s, Aromatic H), 7.38 (2H, s, Aromatic H), 4.49 (2H, s, -OC*H*$_2$COOH), 1.60 (6H, s, 2C*H*$_3$). ^{13}C NMR (DMSO) δ (ppm): 168.75, 149.72, 149.32, 149.10, 143.40, 131.07, 130.48, 117.24, 112.02, 68.87, 41.84, 29.89. MS-ESI: $[M-H]^-$ m/z = 600.8.

6,6-Bis(4-hydroxyphenyl)heptanoic acid (hapten T4): A mixture of phenol (47 g, 0.5 mol), 5-acetylvaleric acid (7.2 g, 0.05 mol), thioglycolic acid (32 mg, 5 mmol) and weakly acidic ion-exchange resin (43.2 g) was heated in an oil bath at 90 °C for 17 h. The mixture was cooled to ambient temperature and 50 mL of ethyl acetate was added. After stirring for 30 min, the solvent was filtered and the filtrate was mixed with 50 mL of saturated solution of sodium bicarbonate. After stirring for 30 min, the aqueous layer was collected, acidified to pH 1–2 with 37% HCl and then extracted with ethyl acetate (2 mL × 25 mL). The organic phase was washed with water (3 mL × 20 mL) and dried overnight with anhydrous sodium sulfate (10 g). The solvent was evaporated under vacuum to obtain a brown oil, which was purified by flash silica column chromatography (ethyl acetate/petroleun ether, 1:4, v/v) to obtain the product 6,6-bis(4-hydroxyphenyl)heptanoic acid (T4). Yield: 54%. ^{1}H NMR (DMSO) δ (ppm): 11.92 (1H, br, COO*H*), 9.11 (2H, s, O*H*), 6.94–6.91 (4H, d, J = 8.64 Hz, Aromatic H), 6.64–6.61 (4H, d, J = 8.64 Hz, Aromatic H), 2.16–2.06 (2H, m, —CH$_2$CH$_2$CH$_2$C*H*$_2$COOH), 1.96–1.91 (2H, m, —C*H*$_2$CH$_2$CH$_2$CH$_2$COOH), 1.51–1.41 (5H, m, —CH(C*H*$_3$)CH$_2$C*H*$_2$—), 1.08–1.00 (2H, m, —CH$_2$CH$_2$C*H*$_2$CH$_2$COOH). ^{13}C NMR (DMSO) δ (ppm): 174.53, 154.92, 140.23, 127.87, 114.69, 44.24, 41.42, 33.84, 27.66, 25.36, 24.20. MS-ESI: $[M-H]^-$ m/z = 313.0.

6,6-Bis(3,5-dibromo-4-hydroxyphenyl)heptanoic acid (hapten T5): Liquid bromine (25.7 g, 0.16 mol) in 10 mL of glacial acetic acid was slowly added into T4 (3.14 g, 10 mmol) in 40 mL of glacial acetic acid during a 50-min period and stirred continuously for 22 h. The mixture was poured into an aqueous saturated solution of sodium bisulfate. The resulting semisolid mass was separated by filtration and washed with cold water. After drying, the semisolid was re-dissolved in 100 mL of methanol under stirring for 30 min. The insoluble matter was removed by filtration and the filtrate was evaporated under vacuum to obtain a crude product, which was purified by flash silica column chromatography (ethyl acetate/*n*-hexane, 1:4, v/v) to get 6,6-bis(3,5-dibromo-4-hydroxyphenyl)heptanoic acid (T5) with a yield of 43%. ^{1}H NMR (DMSO) δ (ppm): 11.94 (1H, br, COO*H*), 9.82 (2H, s, O*H*), 7.28 (4H, s, Aromatic H), 2.52–2.50 (2H, t, J = 1.8 Hz, —CH$_2$CH$_2$CH$_2$C*H*$_2$COOH), 2.20–2.15 (2H, t, J = 7.5 Hz, —C*H*$_2$CH$_2$CH$_2$CH$_2$COOH), 1.49 (3H, s, C*H*$_3$), 1.55–1.45 (2H, m, —CH$_2$CH$_2$C*H*$_2$CH$_2$COOH), 1.05–0.95 (2H, m, —CH$_2$C*H*$_2$CH$_2$CH$_2$COOH). ^{13}C NMR (DMSO) δ (ppm): 174.53, 148.85, 143.25, 130.78, 111.94, 44.62, 33.65, 26.59, 25.05, 23.90. MS-ESI: $[M-H]^-$ m/z = 628.7.

4-(3,5-Dibromo-4-hydroxyphenyl)butanoic acid (hapten T6): 4-(4-methoxyphenyl)butanoic acid (5 g, 25.7 mmol) was melted by heating under the protection of a stream of nitrogen gas and then hydrogen bromide (6.5 mL, 53 mmol) was added dropwise. After stirring for 6 h at 110 °C, the mixture was cooled to 80 °C and 50 mL of water was added. Then, the solution was further cooled in ice-water bath for 1 h. The light red precipitate was collected and recrystallized in ethyl acetate/*n*-hexane to obtain the product 4-(4-hydroxyphenyl)butanoic acid with a yield of 53%. ^{1}H NMR (DMSO) δ (ppm): 11.98 (1H, s, COO*H*), 9.16 (1H, s, O*H*), 7.11–7.08 (2H, d, J = 8.55 Hz, Aromatic H), 6.69–6.67 (2H, d, J = 8.55 Hz, Aromatic H), 2.52–2.50 (2H, t, J = 1.8 Hz, —$CH_2CH_2CH_2$COOH), 2.21–2.16 (2H, t, J = 2.0 Hz, —$CH_2CH_2CH_2$COOH), 1.79–1.69 (2H, m, —$CH_2CH_2CH_2$COOH). ^{13}C NMR (DMSO) δ (ppm): 174.50, 155.55, 131.75, 129.30, 115.25, 33.75, 33.21, 26.78. MS-ESI: $[M-H]^-$ m/z = 178.8.

Liquid bromine (14.1 g, 88 mmol) was slowly added into a mixture of 4-(4-hydroxyphenyl)butanoic acid (2 g, 11 mmol) in 30 mL of glacial acetic acid during a 30-min period and stirred continuously for 20 h. After adding 50 mL of saturated solution of sodium bisulfate, the mixture was cooled in ice-water bath for 2 h. The precipitate was isolated by filtration and recrystallized in ethyl acetate/n-hexane, leading to a white product 4-(3,5-dibromo-4-hydroxyphenyl)butanoic acid (T6). Yield: 48%. ^{1}H NMR (DMSO) δ (ppm): 12.02 (1H, br, COO*H*), 9.67 (1H, s, O*H*), 7.37 (2H, s, Aromatic H), 2.75–2.50 (2H, t, J = 7.83 Hz, —$CH_2CH_2CH_2$COOH), 2.53–2.51 (2H, t, J = 7.8 Hz, —$CH_2CH_2CH_2$COOH), 1.80–1.70(2H, m, —$CH_2CH_2CH_2$COOH). ^{13}C NMR (DMSO) δ (ppm): 174.32, 148.91, 136.36, 132.15, 112.09, 33.10, 32.90, 26.30. MS-ESI: $[M-H]^-$ m/z = 336.7.

2.4. Preparation of immunogens and coating antigens

All haptens containing carboxylic acids were converted to active esters for coupling to carrier proteins [23]. Haptens T1, T3, and T5 were conjugated to KLH for immunogens and all haptens were conjugated to BSA for coating antigens. Conjugates were separated from uncoupled haptens via dialysis against phosphate buffered saline (PBS, 0.01 mol L^{-1} phosphate, 0.137 mol L^{-1} NaCl, and 3 mmol L^{-1} KCl, pH 7.4) at 4 °C for 3 days in the dark with six changes of the dialysis solution. All the conjugates were lyophilized and stored at 4 °C until use.

2.5. Immunization and antiserum preparation

The animal use protocol was approved by the China Agricultural University Animal Care and Use Committee. The immunization procedure followed the protocol reported previously [24]. Each immunogen was injected into three female New Zealand White rabbits. Final serum collection was 4 months following the first immunization. Antiserum was obtained by centrifugation, stored at −20 °C, and used without purification.

2.6. ELISA performance

The preparation of the assay buffers and the performance of the indirect competitive ELISA have been previously described [24]. Optimal concentrations of coating conjugates and serum dilution were selected by checkerboard titration. The dose-response curves of ELISA were generated in SigmaPlot 10.0 and the half-maximum inhibition concentration (IC_{50}) was obtained from the fit of the data with a four-parameter logistic equation.

2.7. Optimization of ELISA

Effects of different variables including organic solvents, pH and ionic strength on assay performance (IC_{50} and A_0, the absorbance in the absence of TBBPA) were studied at ambient temperature. DMSO and methanol were separately added to PBST (PBS plus 0.05% Tween 20) to form the final percentage of 0–30% (v/v). The effect of pH was evaluated using different PBST solutions, with adjusted pH values ranging from 4.9 to 10. To estimate the influence of ionic strength, PBST solutions containing NaCl at concentrations ranging from 0 to 1.09 mol L^{-1} were tested.

2.8. Cross-reactivity study

Specificity of the optimized assay was tested by measuring cross-reactivity (CR) using a group of structural analogs. The CR was calculated as follows: CR (%) = [IC_{50} (TBBPA)/IC_{50} (tested compound)] × 100.

2.9. Sampling and sample preparation

The physico-chemical properties of TBBPA suggest that soil and sediment would be important sinks and, therefore, these matrices were employed for the analysis of TBBPA. All soils and sediments were collected in the Beijing area during May–July 2011 (Fig. 2). Thirteen surface sediment samples were collected along the Qinghe canal. Four surface soil samples were collected from an open e-waste recycling site (around 400 m^2) and eleven soil samples were collected from the plow layer (0–10 cm) of farmlands. All samples were lyophilized, ground, and sieved through a 20-mesh (0.9 mm aperture) screen. These samples were stored in sealed containers at −20 °C until analysis. For the recovery study, samples were spiked with TBBPA in 1 mL of methanol to reach final concentrations of 1.0, 10 and 100 ng g^{-1}, followed by solvent evaporation for 1 h.

2.10. Extraction and cleanup

A dry weight (dw) of 5 g of sample was added into a Soxhlet thimble and extracted with 200 mL of dichloromethane:acetone (1:4, v/v) for 24 h using the conventional procedure. After filtering through a 0.2-μm filter (Waters Corp., MA), the extract was evaporated and the residue was redissolved in 3–5 mL of methanol. The cleanup step followed the procedure reported by Guerra et al. [17] with minor modifications. Briefly, the extract was applied to a C_{18} solid phase extraction (SPE) cartridge (6 mL, Agilent Technologies, Inc.) which was pre-conditioned with 4 mL of dichloromethane and 4 mL of methanol. TBBPA in the cartridge was eluted with 6 mL of methanol. To one volume of the elute two volumes of aqueous HCl solution (1%) and two volumes of dichloromethane were added. After agitation and centrifugation, the organic phase was recovered and washed with deionized water. Under a gentle nitrogen stream, the organic phase was reduced to near dryness and the residue was redissolved with 200 μL of methanol. The extracts of soil and sediment were diluted with PBST at least 20-fold and 50-fold, respectively, prior to ELISA.

The sample extraction and cleanup procedures for the LC–MS/MS method were similar to those described above, except 50 μL of $^{13}C_{12}$-TBBPA solution at a concentration of 5 ng μL^{-1} was spiked into the sample before the extraction. The final extract was filtered through a 0.2-μm filter prior to LC–MS/MS.

3. Results and discussion

3.1. Hapten design and synthesis

To develop a sensitive and specific immunochemical analysis of TBBPA, six haptens (Fig. 1) were synthesized mimicking different structural elements of TBBPA. Three of the haptens were used to prepare antibodies and all of the hapten coupled conjugates were employed as coating antigens since heterologous assays frequently

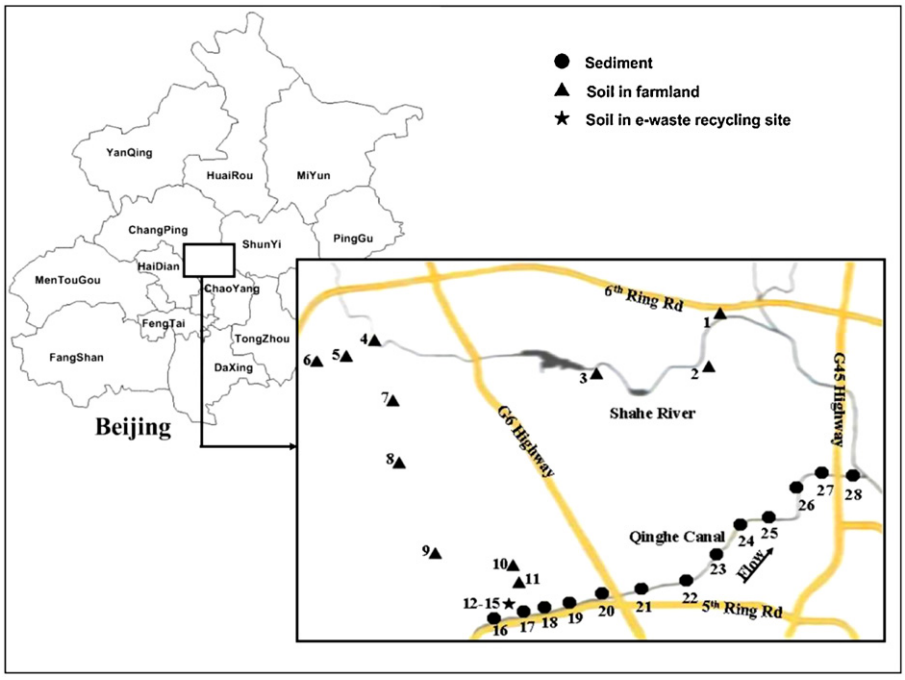

Fig. 2. Location of sampling sites.

lead to improved sensitivity and specificity compared to homologous assays. The phenolic group of TBBPA seemed to be the most convenient group to attach a linker. A common strategy was to convert the hydroxyl group to an ether, with the ether side chain terminated with a carboxylic acid. The acidity of phenolic group makes it easy to generate the corresponding alkoxide with sodium hydroxide; the subsequent reaction with 3-chloropropionic acid leads to the acid T1. Alternatively, the reaction with ethyl bromoacetate, followed by ester hydrolysis, leads to the acid T2 in a good yield. This gave us two haptens with the linker attached to the terminal portion of the molecule with the remainder of the molecule a good mimic for TBBPA. This would allow the testing of both heterologous and homologous assays.

T5 was obtained by coupling two phenols through 5-acetylvaleric acid, leading to the intermediate T4, followed by the substitution of bromine on the positions *ortho* to hydroxyl groups [25]. Attaching the linker to the center of the molecule maintained the TBBPA structure to a major degree, and in particular both the phenolic groups were free, preserving these potentially important binding areas. Hapten T3, commercially available, has a similar structure, but a shorter linker. Hapten T4, from the synthesis of T5, without the substitution of bromine was used to study the effect of bromine substitution on the immunoassay.

The use of fragment-derived haptens has successfully improved the sensitivity of immunoassays by relatively enhancing the affinity of antibody to the analytes [20,22]. Consequently, hapten T6 that only mimics the 3,5-dibromo-4-hydroxyphenyl was designed for this purpose. It was obtained by the demethylation of 4-(4-methoxyphenyl)butanoic acid with a slight excess of aqueous hydrogen bromide, followed by the substitution of bromine on the positions *ortho* to hydroxyl group.

3.2. Antiserum screening

During the immunization, two rabbits (one receiving KLH-T1 and another receiving KLH-T3) died. The seven antisera collected were titrated in homologous and heterologous conjugate-coated format ELISA. All of the antisera showed high titers with homologous conjugates and no significant affinity for BSA-T4 and BSA alone (data not shown). The failure of the antisera to show any affinity for T4 demonstrated the importance of the bromine atoms in producing binding. The combinations of antisera/coating conjugates that showed specific recognition were used to carry out competitive assays. The antisera against haptens T1, T3, and T5 consistently had IC_{50} values ranging from 1.0 to 15 ng mL^{-1} with both homologous and heterologous conjugates (Table 1), suggesting that these immunizing haptens are the close mimics of TBBPA. The superiority of the T1 based antisera suggested the hapten is better linked to the protein via the end of the molecule rather than the middle of the molecule as in the T3 and T5 based antisera. The superiority of T5 vs T3 suggested the longer linker allows the antibody to better recognize the TBBPA structural elements since these would be further from the linked protein. The best sensitivity to TBBPA (IC_{50} = 1.0 ng mL^{-1}) was observed in the combination of KLH-T1/BSA-T2 (Table 1), which was selected for further ELISA development. The T1 based immunogen showed good response to all of the coating antigens with the T2 based coating antigen being slightly better than the others. The T6 coating antigen represented only the terminal fragment of the TBBPA structure but showed very good sensitivity indicating this portion of TBBPA was critical to having good binding characteristics for the assay using the T1 antiserum. Interestingly, the T6 coating antigen did not perform well

Table 1
IC_{50} values of TBBPA (ng mL^{-1}) by ELISA with different combinations of antiserum and coating antigen.

Antiserum against different immunogens[a]	Coating antigens				
	BSA-T1	BSA-T2	BSA-T3	BSA-T5	BSA-T6
KLH-T1	1.4	1.0	3.2	3.8	1.2
KLH-T3	4.6	3.0	1.8	2.5	15
KLH-T5	3.5	4.1	1.2	1.7	12.3

[a] For each immunogen, only data from the antiserum having lowest IC_{50} values by ELISA are shown.

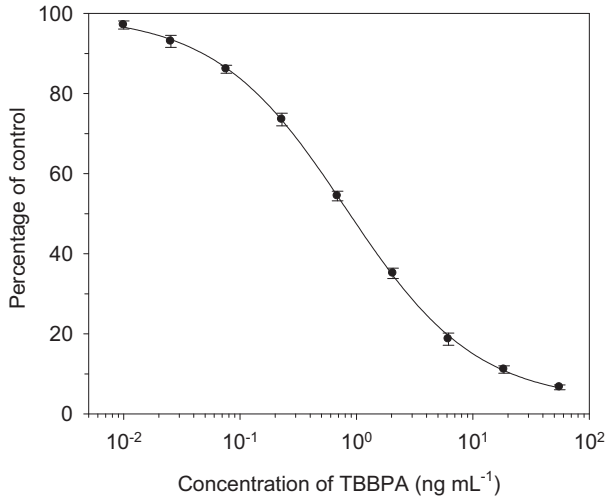

Fig. 3. Calibration curve of indirect ELISA for TBBPA. Plates were coated with 6 ng BSA-T2 per well and the antiserum against KLH-T1 was diluted 40,000-fold in PBST. The data are average of six replicates. Error bars indicate standard deviations from six replicate measurements.

with antisera derived from haptens coupled to the center of the molecule. The ELISAs using antisera from haptens coupled through the center of the molecule and coating antigens derived from haptens coupled in the same manner showed lower IC_{50} values. The difference between heterologous and homologous was not great providing the haptens were from the same series although heterologous coating antigens were slightly better for two out of the three cases examined here.

3.3. ELISA optimization

Varying percentages of DMSO and methanol in the assay buffer were first studied because the solvents have been reported to be efficient solubilizers for lipophilic compounds in assay buffer [20,22]. There were no significant effects on the IC_{50} and A_0 values with methanol or DMSO percentages in the range of 0–10%. Methanol is often used in the sample pretreatment for TBBPA and, thus, 10% methanol-PBST was selected to prepare standard and sample solutions due to the greater sensitivity (lower IC_{50}, 0.9 ng mL^{-1}) and the reasonable A_0 (0.92 A.U.).

As buffer pH values varied between 4.9 and 10, the IC_{50} and A_0 values changed in the ranges of 0.85–1.5 ng mL^{-1} and 0.78–0.92 A.U., respectively. Although the IC_{50} showed minimal changes between 5.9 and 8, a minimum IC_{50} (maximum sensitivity) occurred at 7.4. The best combination IC_{50} and A_0 (IC_{50} = 0.85 ng mL^{-1} with A_0 = 0.92) was obtained at pH 7.4, and, therefore, this pH was selected for further optimization assays. Finally, 0.137 mol L^{-1} NaCl was employed for the PBST preparation as the best combination of IC_{50} and A_0 (IC_{50} = 0.86 ng mL^{-1} with A_0 = 0.89) was obtained at this concentration.

Fig. 3 is a typical dose-response curve of TBBPA under the optimized condition of PBST (0.137 mol L^{-1} NaCl, pH 7.4) containing 10% methanol. This assay has an IC_{50} value of 0.87 ng mL^{-1} and an IC_{10} value of 0.05 ng mL^{-1}, which was defined as the limit of detection (LOD) in the buffer system.

3.4. Cross-reactivity study

Assay selectivity was evaluated using a set of important BFRs including tetrabromobisphenol A-bis(2,3-dibromopropylether) (TBBPA-DBPE), 2,2′,6,6′-tetrabromobisphenol A diallyl ether (TBBPA-BAE), HBCD, 1,2-bis(pentabromodiphenyl) ethane (DBDPE), 1,2-bis(2,4,6 tribromophenoxy) ethane (BTBPE), bis(2-ethylhexyl)-3,4,5,6-tetrabromophthalate (TBPH), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), and PBDEs. This assay is specific for TBBPA as demonstrated by the low cross-reactivity (<0.05%) to the tested compounds (Table 2). Specific antibodies for a given compound can be elicited from the haptens mimicking its structure, electronic properties, and hydrophobicity. These results indicate that the tested immunogens are valuable for eliciting high-affinity antibodies to TBBPA, suitable for its specific detection at low levels.

Table 2
Cross-reactivity of the antiserum with TBBPA structural analogs.

Compounds	CR (%)
TBBPA	100
TBBPA-BDPE	<0.05
TBBPA-BAE	<0.05
HBCD	<0.02
DBDPE	<0.02
BTBPE	<0.02
TBPH	<0.02
TBB	<0.02
BPA	<0.02
BDE-28	<0.05
BDE-47	<0.05
BDE-99	<0.01
BDE-100	<0.01
BDE-153	<0.01
BDE-154	<0.01
BDE-183	<0.01
BDE-209	<0.01
5-OH-BDE-47	<0.05
5-MeO-BDE-47	<0.05

3.5. Matrix effect

To evaluate the matrix effect on the assay performance, the final extracts from the blank (unspiked) samples were diluted with PBST and then used to generate the dose-response curves, which were compared with that generated in PBST. Overlapping curves were obtained when the soil and sediment extracts were diluted at least 20-fold and 50-fold, respectively (data not shown), indicative of higher matrix effects from sediment extracts. Taking into account of the required dilution factor to minimize matrix interference, the LODs of this method for TBBPA in soil and sediment were approximately 0.04 ng g^{-1} and 0.1 ng g^{-1} dw, respectively.

3.6. Recovery

TBBPA is likely to be associated tightly with the organic matters in soils and sediments, due to its low solubility in water and a high log K_{ow} (4.5–5.3) [26]. Soxhlet extraction, a robust and efficient technique, is a primary option for the extraction of TBBPA in soils and sediments. The average recoveries and coefficient of variant (CV) values of ELISA for TBBPA in spiked samples were in the range of 93–117% and 4–11%, respectively, whereas the LC–MS/MS gave recoveries of 88–107% and CVs of 3–8% (Table 3). The ELISA results tended to be slightly higher than those of LC–MS/MS method. The recoveries and CVs of ELISA are acceptable for screening purposes.

3.7. Determination of TBBPA in real world samples

The ELISA method was applied to analyze TBBPA in real world samples including 15 soils and 13 sediments. The levels of TBBPA in soils showed great variations dependent on the nature of the site. As expected the concentrations of TBBPA detected in soils from the e-waste recycling site were quite high ranging from 26 to 104 ng g^{-1}, whereas, the levels of TBBPA in farmland soils were low, being below the LOD (0.04 ng g^{-1}) in all except two samples, #5 and #11

Table 3
Determination of TBBPA in spiked samples by ELISA and LC–MS/MS.

Sample	TBBPA spiked ($ng\,g^{-1}$, dw)	Average recovery[a] (%) ± CV% ($n = 3$)	
		ELISA	LC–MS/MS
Soil	0	0 ± 0	0 ± 0
	1.0	112 ± 8	107 ± 6
	10	96 ± 7	95 ± 3
	100	103 ± 4	98 ± 4
Sediment	0	0 ± 0	0 ± 0
	1.0	93 ± 11	88 ± 8
	10	117 ± 6	90 ± 5
	100	109 ± 9	104 ± 4

[a] TBBPA found in blank (unspiked) sediment sample by ELISA and LC–MS/MS (0.8 $ng\,g^{-1}$ dw and 0.6 $ng\,g^{-1}$ dw, respectively) were subtracted for the calculation of recoveries.

at 5.6 $ng\,g^{-1}$ and 0.8 $ng\,g^{-1}$, respectively (Table 4). TBBPA can be easily released into the environment due to improper handling and disposal of e-wastes, which can potentially pose hazards to human health and create severe environmental problems [27]. He et al. [28] reported the elevated concentrations of TBBPA were found in birds collected from an e-waste recycling region in South China, because of the continual exposure caused by e-waste recycling activities. The presence of TBBPA in the farmland soils could be due to the recent input of compost, which can be a major input of organic pollutants to the soil [29]. A recent study reported that plants, e.g., cabbage and radish, can absorb TBBPA from soil posing a risk to humans consuming these plants [30].

TBBPA was detectable in all the sediments, with an average concentration of 7.7 $ng\,g^{-1}$ dw (0.3–22 $ng\,g^{-1}$ dw) (Table 4). The canal from which the sediments were sampled is an approximately 20-km-long waterway that receives drainage from the area along the north 5th ring road of Beijing city. As a result of the inflow of widespread runoff, the discharge of sewage treatment plants, and direct dumping of household and yard wastes, the canal was consequentially contaminated with BFRs. Elevated levels of TBBPA were observed in the samples from #16 to #22 (5.0–22 $ng\,g^{-1}$ dw), probably due to the vicinity of these sampling sites to the downtown and the upstream e-waste site. In our recent study [22], BDE-47 equivalents were found in the sediment from the same canal using an ELISA method, with an average concentration of 24 $ng\,g^{-1}$ dw (1.4–55 $ng\,g^{-1}$ dw), higher than TBBPA levels detected in this study. This is consistent with the fact that TBBPA is used primarily as a reactive flame retardant and as such its release from treated goods is likely to be less facile than for PBDEs whose use pattern is largely or exclusively as additive flame retardants.

Table 4
Concentrations of TBBPA in real world samples determined by ELISA.

Sample No.	Location	TBBPA concentration ($ng\,g^{-1}$, dw), $n = 3$
Soils from farmlands		
#1	N40°09.464, E116°24.351	<LOD
#2	N40°07.856, E116°23.997	<LOD
#3	N40°07.677, E116°19.847	<LOD
#4	N40°08.580, E116°11.756	<LOD
#5	N40°08.172, E116°10.758	5.6 ± 0.5
#6	N40°08.054, E116°09.604	<LOD
#7	N40°06.887, E116°12.405	<LOD
#8	N40°05.171, E116°12.617	<LOD
#9	N40°02.642, E116°13.935	<LOD
#10	N40°02.287, E116°16.830	<LOD
#11	N40°01.710, E116°16.882	0.8 ± 0.05
Soils from the e-waste recycling site		
#12	N40°00.897, E116°16.860	104 ± 8.8
#13	N40°00.890, E116°16.852	26 ± 1.4
#14	N40°00.905, E116°16.871	68 ± 6.4
#15	N40°00.892, E116°16.855	73 ± 6.5
Sediments		
#16	N40°00.653, E116°16.117	21 ± 1.5
#17	N40°00.796, E116°17.157	14 ± 0.9
#18	N40°00.928, E116°17.962	5.0 ± 0.7
#19	N40°01.124, E116°18.898	22 ± 1.0
#20	N40°01.466, E116°20.446	9.2 ± 0.3
#21	N40°01.665, E116°21.555	18 ± 1.6
#22	N40°01.852, E116°23.012	6.6 ± 0.6
#23	N40°02.616, E116°24.194	2.1 ± 0.2
#24	N40°03.425, E116°25.051	0.3 ± 0.02
#25	N40°03.608, E116°26.157	1.3 ± 0.1
#26	N40°04.442, E116°27.112	4.5 ± 0.4
#27	N40°04.812, E116°28.001	0.9 ± 0.1
#28	N40°04.721, E116°29.424	3.3 ± 0.3

3.8. Comparison to other studies

The comparison to other studies can help us know the pollution pattern of TBBPA, despite differences in methodology and in the sites themselves. Generally, levels of TBBPA in environmental matrices were low except samples from locations near industrial emission sites (Table 5). TBBPA levels (0.2–22 $ng\,g^{-1}$ dw) found in the sediments of this study were much lower than those from the downstream of a plastic factory using TBBPA in Sweden (270 $ng\,g^{-1}$ dw) [31] or close to a BFR manufacturing site in UK (9.8 $\mu g\,g^{-1}$ dw) [32]. The results of this study are comparable to TBBPA concentrations measured in matrices from locations not directly influenced by industrial emissions, such as sediments from the Scheldt basin (0.1–67 $ng\,g^{-1}$ dw) [32], the Western Scheldt (0.1–3.2 $ng\,g^{-1}$ dw) [32], Dutch rivers (0.1–6.9 $ng\,g^{-1}$ dw) [32], Spanish rivers (0–15 $ng\,g^{-1}$ dw) [17], the Detroit river (0.6–1.84 $ng\,g^{-1}$ dw) [33], lake Erie (<0.05–0.51 $ng\,g^{-1}$ dw) [34] and the Japanese Neya river (20 $ng\,g^{-1}$ dw) [35] and Osaka bay (0.7–3.1 $ng\,g^{-1}$ dw) [36]. TBBPA was also found in various Spanish soil samples at levels ranging from 3.4 to 32.2 $ng\,g^{-1}$ in industrial soils and from undetectable to 0.3 $ng\,g^{-1}$ in agricultural soils [37]. These concentrations are within the range of TBBPA levels of soils in the present study.

Although TBBPA has been known to be an ubiquitous environmental contaminant, little data on TBBPA in both abiotic and biotic matrices is available in China [15,16,28,38–44]. High levels of TBBPA found in Chinese environmental matrices were expectantly related to the locations near TBBPA emission sources (Table 5), e.g., soils near a garbage dumping site (1.36–1.78 $\mu g\,g^{-1}$) [41], a TBBPA manufacturing plant (156 $ng\,g^{-1}$) [39] and an e-waste recycling site in this study (104 $ng\,g^{-1}$); and sediments close to a TBBPA manufacturing plant (806 $ng\,g^{-1}$ dw) [39], a seriously contaminated site of Chaohu lake (482 $ng\,g^{-1}$ dw) [44] and an industrial zone (230 $ng\,g^{-1}$ dw) [15]. The soils and sediments even far from the industrial emission have been contaminated with TBBPA although the levels are currently low (Table 5). The presence of TBBPA, even at low levels, demonstrates its release into the environment. As the TBBPA demand rises in China, its transfer to the environment may increase in the future. More studies need to be carried out to determine the environmental fate of TBBPA around China and throughout the world. This ELISA is hopefully a valuable tool.

3.9. Comparison of ELISA with LC–MS/MS

To evaluate the applicability of the developed ELISA for TBBPA, the results of this assay were compared with those of LC–MS/MS method for 16 sediments and soils (Fig. 4). The average concentrations of TBBPA obtained by ELISA were slightly higher than those by LC–MS/MS, but not statistically significant according to a Paired t-test ($P > 0.05$). The slight discrepancy could be due to the matrix

Table 5
Comparison of TBBPA contamination of sediment and soil samples in different studies.

Matrix	Sampling location	Sampling year	Method	TBBPA conc. (ng g^{-1}, dw)	Study
Sediment	Downstream of a plastic factory, Sweden	Not indicated	GC-NCI-MS	270	[31]
	Upstream of a plastic factory, Sweden	Not indicated	GC-NCI-MS	34	[31]
	Rivers in UK	2000 and 2002	LC-ESI-MS	<2.4–9750	[32]
	Scheldt basin, Belgium	2000	LC-ESI-MS	<0.1–67	[32]
	Western Scheldt, The Netherlands	2000	LC-ESI-MS	<0.1–3.2	[32]
	Rivers, The Netherlands	2000	LC-ESI-MS	<0.1–6.9	[32]
	Rivers in Spain	2006 and 2008	LC-ESI-MS/MS	0–15	[17]
	Detroit river, US	2000	GC-HRMS	0.6–1.84	[33]
	Lake Erie, Canada	2004	LC-ESI-MS/MS	<0.05–0.51	[34]
	Neya river, Japan	1981	GC-ECD-MS	20	[35]
	Osaka bay, Japan	1999	HRGC-HRMS	0.7–3.1	[36]
	A river near TBBPA plants, China	2005	UPLC-ESI-MS/MS	806	[39]
	Dongjiang river, China	2006	GC-SIM-MS	3.8–230	[15]
	Chaohu lake, China	2008	HPLC-PDA	22–482	[44]
	A canal in Beijing, China	2011	ELISA	0.2–22	This study
Soil	Industrial region, Spain	Not indicated	GC-SIM-MS	3.4–32.2	[37]
	Agricultural fields, Spain	Not indicated	GC-SIM-MS	0.3	[37]
	A garbage dumping site, China	Not indicated	HPLC-UV	1360–1780	[41]
	Outside TBBPA manufacturing plants, China	2005	UPLC-MRM-MS/MS	0.12 (ng g^{-1} wet weight)	[38]
	Outside TBBPA manufacturing plants, China	2005	UPLC-ESI-MS/MS	25.2 ± 2.7 ($n = 4$)	[40]
	Garden at campuses, China	2005	UPLC-ESI-MS/MS	Undetectable	[40]
	An e-waste recycling site in Beijing, China	2011	ELISA	16–134	This study
	Farmland in Beijing, China	2011	ELISA	<0.04–5.6	This study

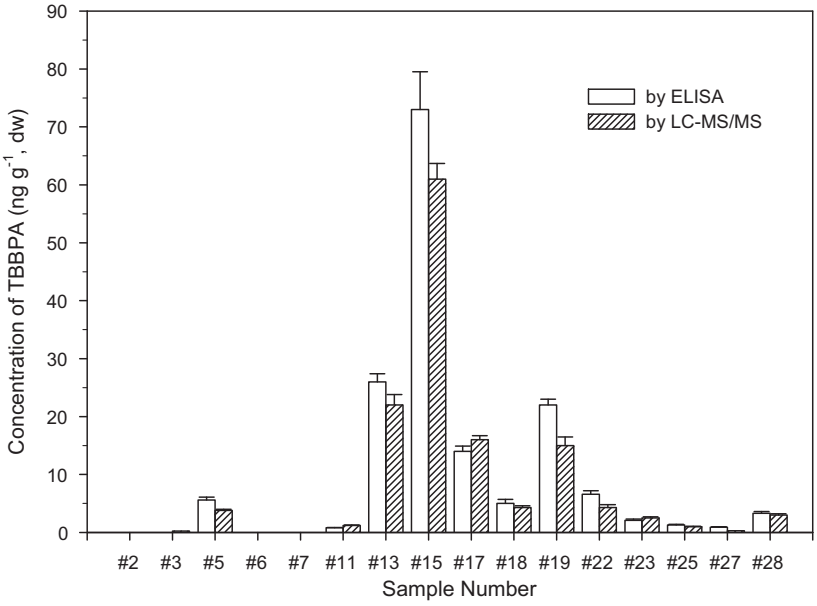

Fig. 4. Comparison of the results of ELISA and LC–MS/MS methods for TBBPA in real world samples. The data are average values of triplicate samples. Error bars indicate standard deviations from triplicate measurements.

effects or cross-reactivity of unknown compounds in the extracts by ELISA method.

4. Conclusions

This is the first sensitive and selective ELISA for TBBPA. An excellent antiserum was produced using the immunogen of which the hapten has a propanoic acid linker via a hydroxyl at the terminal position of TBBPA. A heterologous coating hapten having an acetic acid spacer attached to the same position resulted in the highest assay sensitivity. This assay showed an IC_{50} of 0.87 ng mL^{-1} for TBBPA, with negligible cross-reactivity (<0.05%) to a number of structural analogs. The recovery and coefficient of variation of this assay for TBBPA in soil and sediment samples after Soxhlet extraction, simple cleanup and concentration steps were acceptable. TBBPA was found in sediments and soils in Beijing area not directly influenced by industrial emissions, indicating its multiple transfer processes and sources. A comparison between the ELISA and the LC–MS/MS methods for real samples showed good correlation demonstrating this ELISA method would make an excellent screening tool prior to using more resource intensive methods needed for the analysis of TBBPA.

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References

[1] Bromine Science and Environmental Forum. http://www.bsef.com, 2001 (accessed 06.04.12).
[2] European Brominated Flame Retardant Industry Panel (EBFRIP), Statement June 4, 2007.
[3] Y.Q. Jiang, Chin. J. Flame Retardant Mater. Technol. 2 (2007) 1–7 (in Chinese).
[4] S.J. Munn, R. Allanou, K. Aschberger, O. Cosgrove, S. Pakalin, A. Paya-Perez, G. Pellegrini, B. Schwarz-Schulz, S. Vegro, Luxembourg Office for Official Publications of the European Communities, 2006, pp. 35–114.
[5] J.B. Fini, A. Riu, L. Debrauwer, A. Hillenweck, S.L. Mével, S. Chevolleau, A. Boulahtouf, K. Palmier, P. Balaguer, J.P. Cravedi, B.A. Demeneix, D. Zalko, Toxicol. Sci. 125 (2012) 359–367.
[6] E.C. Kibakaya, K. Stephen, M.M. Whalen, J. Immunotoxicol. 6 (2009) 285–292.
[7] H. Viberg, P. Eriksson, Toxicology 289 (2011) 59–65.
[8] J.M. McCormick, M.S. Paiva, M.M. Häggblom, K.R. Cooper, L.A. White, Aquat. Toxicol. 100 (2010) 255–262.
[9] K.W. George, M.M. Haggblom, Environ. Sci. Technol. 42 (2008) 5555–5561.
[10] T. An, L. Zu, G. Li, S. Wan, B. Mai, P.K. Wong, Bioresource Technol. 102 (2011) 9148–9154.
[11] H. Okada, T. Tokunaga, X. Liu, S. Takayanagi, A. Matsushima, Y. Shimohigashi, Environ. Health Perspect. 116 (2008) 32–38.
[12] A. Covaci, S. Voorspoels, M.A.E. Abdallah, T. Geens, S. Harrad, R.J. Law, J. Chromatogr. A 1216 (2009) 346–363.
[13] X.J. Luo, S.J. Chen, B.X. Mai, J.M. Fu, Sci. China Chem. 53 (2010) 961–973.
[14] Z. Xie, R. Ebinghaus, R. Lohmann, O. Heemken, A. Caba, W. Püttmann, Anal. Chim. Acta 584 (2007) 333–342.
[15] X.L. Zhang, X.J. Luo, S.J. Chen, J.P. Wu, B.X. Mai, Environ. Pollut. 157 (2009) 1917–1923.
[16] Z.X. Shi, Y.N. Wu, J.G. Li, Y.F. Zhao, J.F. Feng, Environ. Sci. Technol. 43 (2009) 4314–4319.
[17] P. Guerra, E. Eljarrat, D. Barceló, Anal. Bioanal. Chem. 397 (2010) 2817–2824.
[18] W.L. Shelver, Y.-S. Keum, H.-J. Kim, D. Rutherford, H.H. Hakk, Å. Bergman, Q.X. Li, J. Agric. Food Chem. 53 (2005) 3840–3847.
[19] W.L. Shelver, C.D. Parrotta, R. Slawecki, Q.X. Li, M.G. Ikonomou, D. Barceló, S. Lacorte, F.M. Rubio, Chemosphere 73 (2008) S18–S23.
[20] K.-C. Ahn, S.J. Gee, H.-J. Tsai, D. Bennett, M.G. Nishioka, A. Blum, E. Fishman, B.D. Hammock, Environ. Sci. Technol. 43 (2009) 7784–7790.
[21] H.-J. Kim, M.A. Rossotti, K.-C. Ahn, G.G. González-Sapienza, S.J. Gee, R. Musker, B.D. Hammock, Anal. Biochem. 401 (2010) 38–46.
[22] J. Wang, H. Li, W.L. Shelver, Z. Wang, Q.X. Li, J. Li, T. Xu, Anal. Bioanal. Chem. 401 (2011) 2249–2258.
[23] D.P. McAdam, A.S. Hill, H.L. Beasley, J.H. Skerritt, J. Agric. Food Chem. 40 (1992) 1466–1470.
[24] T. Xu, X. Shao, Q.X. Li, Y.-S. Keum, H. Jing, W. Sheng, J. Li, J. Agric. Food Chem. 55 (2007) 3764–3770.
[25] R.M. Patel, D.P. Carew, J.L. Lach, J. Pharm. Sci. 56 (1967) 1326–1328.
[26] IPCS (International Programme on Chemical Safety). Environmental Health Criteria 172. http://www.inchem.org/documents/ehc/ehc/ehc172.htm 1995 (accessed 06.04.12).
[27] H.G. Ni, H. Zeng, S. Tao, E.Y. Zeng, Environ. Toxicol. Chem. 29 (2010) 1237–1247.
[28] M.J. He, X.J. Luo, L.H. Yu, J. Liu, X.L. Zhang, S.J. Chen, D. Chen, B.X. Mai, Environ. Sci. Technol. 44 (2010) 5748–5754.
[29] R.C. Brändli, T. Kupper, T.D. Bucheli, M. Zennegg, S. Huber, D. Ortelli, J. Müller, C. Schaffner, S. Iozza, P. Schmid, U. Berger, P. Edder, M. Oehme, F.X. Stadelman, J. Tarradellas, J. Environ. Monit. 9 (2007) 465–472.
[30] Y. Li, Q. Zhou, Y. Wang, X. Xie, Chemosphere 82 (2011) 204–209.
[31] U. Sellström, B. Jansson, Chemosphere 31 (1995) 3085–3092.
[32] S. Morris, C.R. Allchin, B.N. Zegers, J.J.H. Haftka, J.P. Boon, C. Belpaire, P.E.G. Leonards, S.P.J. van Leeuwen, J. de Boer, Environ. Sci. Technol. 38 (2004) 5497–5504.
[33] S.C. Quade, M. Alaee, C. Marvin, R. Hale, K.R. Solomon, N.J. Bunce, A.T. Fisk, Organohalogen Compd. 62 (2003) 327–330.
[34] S. Chu, J.J.H. Ciborowski, H. Ahmad, D.G. Haffner, K.G. Drouillard, R. Letcher, Organohalogen Compd. 67 (2005) 847–850.
[35] I. Watanabe, T. Kashimoto, R. Tatsukawa, Bull. Environ. Contam. Toxicol. 31 (1983) 48–52.
[36] S. Ohta, T. Nakao, H. Nishimura, T. Okumura, O. Aozasa, H. Miyata, Organohalogen Compd. 57 (2002) 57–60.
[37] C. Sánchez-Brunete, E. Miguel, J.L. Tadeo, J. Chromatogr. A 1216 (2009) 5497–5503.
[38] J. Jin, H. Peng, Y. Wang, R. Yang, J. Cui, Organohalogen Compd. 68 (2006) 85–88.
[39] J. Jin, H. Peng, Y. Wang, W. Liu, R. Yang, BFR2007 Abstract: Analysis. http://www.bfr2010.com/abstract-download/2007/p. 005.pdf (accessed 06.04.12).
[40] H. Peng, J. Jin, Y. Wang, W.Z. Liu, R.M. Yang, Chin. J. Anal. Chem. 35 (2007) 549–551 (in Chinese).
[41] C. Yu, B. Hu, J. Chromatogr. A 1160 (2007) 71–80.
[42] X.L. Zhang, X.J. Luo, S.J. Chen, B.X. Mai, Chin. J. Anal. Chem. 37 (2009) 1577–1582.
[43] X.J. Luo, X.L. Zhang, S.J. Chen, B.X. Mai, Mar. Pollut. Bull. 60 (2010) 718–724.
[44] P.Q. Zhang, Y.W. Li, J.Y. Li, Y.F. Li, Inner Mongolia Sci. Technol. Econ. 231 (2011) 51–55 (in Chinese).